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STRATEGIC ISOLATION AND SCREENING OF LACCASE-PRODUCING MICROBES IN SOLID-STATE AND SUBMERGED FERMENTATION FOR SUSTAINABLE DYE DECOLORIZATION

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ABSTRACT

Laccase, a polyphenol oxidase enzyme, is prominent for its catalytic potential in oxidizing various aromatic compounds, including the complete decomposition of wood elements. This enzyme is naturally prevalent among plants, insects, microbes, and fungi and is essential for several biotechnological applications due to its high redox potential, notably in dye degradation, wastewater treatment, and soil detoxification. Our study focused on isolating, screening, and increasing laccase production from specifically chosen bacterial and fungal sources. We identified nine bacterial and three fungal strains from various polluted sites and natural environments. These strains were cultivated, with laccase activity measured through ABTS assays for bacteria and GUAIACOL assays for fungi, leading to important findings on enzyme activities. The laccase was further refined using a nutrient medium with added tannic acid and partially purified by ammonium sulphate precipitation. This purified form displayed the capability to decolorize Azo dyes efficiently. Lastly, we evaluated the efficacy of laccase in breaking down dyes and their future use in the decolorization of parchment paper, indicating a promising field of application for this microbial synthesized laccase enzyme.

KEYWORDS: Laccase, Fermentation, ABTS, GUAIACOL, Dye Decolorization

INTRODUCTION

Laccase (EC 1.10.3.2, benzenediol oxygen oxidoreductase), a multinuclear copper-containing enzyme, plays essential roles in melanization, wound healing, and immunity. Found in plants, fungi, bacteria, insects, and crustaceans, it is vital for lignin degradation, crucial in pulp and paper industries. Conventional delignification methods release pollutants, but laccase offers an eco-friendly alternative (Eugino & Sobrinho, 2008; Lara et al., 2003). Besides lignin, laccase oxidizes substrates like aromatic amines and ABTS (Zille et al., 2003). Notable producers include fungi (Deuteromycetes, Ascomycetes, Basidiomycetes) and bacteria such as Azospirillum lipoferum, Streptomyces spp., and Bacillus subtilis (Bugg et al., 2011; Demissie & Kumar, 2014; Naz et al., 2015). Applications extend to bioremediation, biofuel production, biosensors, organic synthesis, and textile dye decolorization (Dubé et al., 2008; Ramsay & Goode, 2004). To meet the current global demand for laccase, the search for laccase-producing species, especially bacteria, is increasing, as they can produce more laccase in a shorter time compared to traditional methods. This ensures a daily supply of the desired products for various business applications. Through genetic engineering, it is also possible to generate enzymes with enhanced thermal stability and other characteristics. The current study aimed to identify the best physiological conditions for laccase synthesis, isolate and describe native bacterial and fungal isolates that could manufacture laccases from various sources, and examine the ability of the laccases produced to decolorize dyes.

MATERIALS AND METHODS:

Collection of Samples

For the cultivation of bacteria, various samples were collected from different locations. A rice rhizosphere soil sample (Lat. 21.14639° N, Long. 72.83500° E) was chosen for the isolation of laccase-producing bacteria and fungi. These samples were collected in clean, sterile, and dry polythene bags from a soil in Pandesara, Udhana, Surat, Gujarat. Additionally, mangrove plant and marine water (Lat. 20.80751° N, Long. 72.84565° E) and soil samples were collected in clean, sterile, and dry polythene bags and bottles from Onjal (Machhivad) Navsari. Other samples, including garden soil, agricultural soil, wheat root, and mushroom samples, were collected from various locations. Effluent and soil sediment samples were gathered from chemically contaminated sites. All collected samples were stored at 4°C.

Isolation of Laccase-Producing Bacteria

The collected soil and water samples were serially diluted from 10^{-1} to 10^{-6} and spread on nutrient agar plates containing 0.1% (w/v) tannic acid media. These plates were incubated at 37°C under aerobic conditions for 24-48 hours to obtain colonies. After 24 hours of incubation, bacterial colonies were observed on nutrient agar plates containing 0.1% tannic acid. Plates were examined for morphologically distinct bacterial isolates (Mukhtar et al., 2019).

Screening of Laccase-Producing Bacteria

Isolates were initially screened for laccase production by plating them on nutrient agar supplemented with 0.1% tannic

acid. The development of a brown color zone surrounding the bacterial growth indicated laccase production. Colonies with brown color zones were selected for secondary screening. For secondary screening, a loopful of bacterial suspension was inoculated in 50 ml of sterile nutrient broth supplemented with 0.1% tannic acid and incubated at 30°C at 125 rpm. Laccase activity was checked daily. Selected bacterial isolates were streaked on nutrient agar plates for pure bacterial isolates and incubated at 37°C for 24 hours (Sharma et al., 2019).

Bacterial Laccase Enzyme Activity Assay (ABTS Assay)

Laccase activity was measured using ABTS {2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)} as a substrate. The reactive mixture consisted of 1.5 ml sodium acetate buffer (1 mM, pH 5.0), 1.5 ml ABTS (0.5 mM), and 1.5 ml cell-free supernatant. The absorbance was recorded at 420 nm using a UV/Visible spectrophotometer (Shimadzu-UV-3600 Plus). One unit of enzyme activity was defined as 1 micromole of ABTS oxidized per minute (Bourbonnais & Paice, 1990).

Isolation of Laccase-Producing Fungi

Soil and mushroom samples (Lat. 20.92134° N, Long. 72.95129° E) were collected from a farm near Vijalpor, Italva, Navsari, using the standard serial dilution method. Laccase-producing fungi were screened based on growth on potato dextrose agar (PDA) media containing specific substrates such as tannic acid and guaiacol. Plates were observed for growth and the development of brown-colored precipitates in tannic acid-containing plates and reddish halo zones in guaiacol-containing plates (Gochev & Krastanov, 2007).

Guaiacol Assay Method for Laccase Activity

Guaiacol was used as a substrate for laccase assay. The reaction mixture contained 3 ml sodium acetate buffer (10 mM, pH 5.0), 1 ml guaiacol, and 1 ml enzyme source. The mixture was incubated at 30°C for 15 minutes, and absorbance was read at 450 nm using a UV spectrophotometer. Enzyme activity was calculated using the extinction coefficient of guaiacol (12,100 M^{-1} cm^{-1}) at 450 nm (Kalra et al., 2013).

Selection of Production Medium:

Bacterial Laccase Production Under Submerged Fermentation

Laccase production was carried out under submerged conditions in a medium containing tryptone (10 g/L), yeast extract (5 g/L), sodium chloride (10 g/L), fructose (0.1 g/L), and copper sulfate (0.001 g/L). Isolates from secondary screening were inoculated in the medium and incubated at 30°C at 120 rpm. Laccase activity was measured (Desai, 2020).

Fungal Laccase Production Submerged and Solid-State Fermentation

For submerged fermentation, the potent strain was cultivated in Olga liquid medium containing various nutrients and kept in an incubator shaker at 200 rpm, 30ºC for 12 days. Samples were collected from the fourth day, and fungal mycelium was separated from the broth by filtering through Whatman No. 1 filter paper. The filtrate was used for enzyme assay (Niladevi & Prema, 2008).

For solid-state fermentation, rice bran moistened with distilled water was incubated with the selected organism at 30ºC for 8 days. Crude culture filtrate was obtained by adding distilled water to the plates and filtering through muslin cloth. The supernatant was used for enzyme assay. (Niladevi & Prema, 2008).

Partial Purification of Bacterial and Fungal Laccase Enzyme

The supernatant of all isolated bacterial and fungal laccase enzyme was saturated by 80% ammonium sulfate to recover the extracellular protein enzyme and centrifuged. The pellet was dissolved in phosphate buffer and dialyzed using dialysis membrane-60. The dialyzed enzyme product was kept at 4°C (Majolagbe et al., 2012).

Laccase Application in Decolorization of Azo Dyes

All dialyzed laccase enzyme products were used for dye decolorization, with the reaction mixture containing phosphate buffer, enzyme, and Congo dye at various concentrations. Decolorization was monitored spectrophotometrically at 490 nm (Molina et al., 2009).

RESULTS

In the present study, enhanced samples were streaked on nutrient agar plates and incubated for 24-48 hours at 37°C. Out of 9 different bacterial colonies plated on nutrient agar supplemented with 0.01% tannic acid, 6 distinct colonies showed a brownish zone indicating tannic acid oxidation. These colonies were subjected to secondary screening in a liquid medium for laccase production. Bacterial isolate FSNCO exhibited the highest laccase activity of 0.0.72 U/ml and was selected for further analysis. Identified based on cultural and biochemical characteristics, this isolate was further examined using ABTS, confirming positive laccase activity, with the highest activity observed in the bacterial strain Pseudomonas spp. isolated from wheat roots.

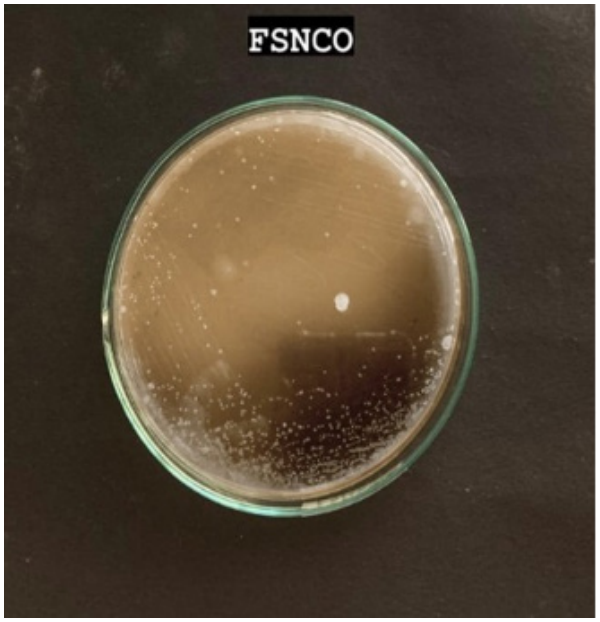

Fig. 1 Bacterial Isolate -FNSCO- Pseudomonas spp

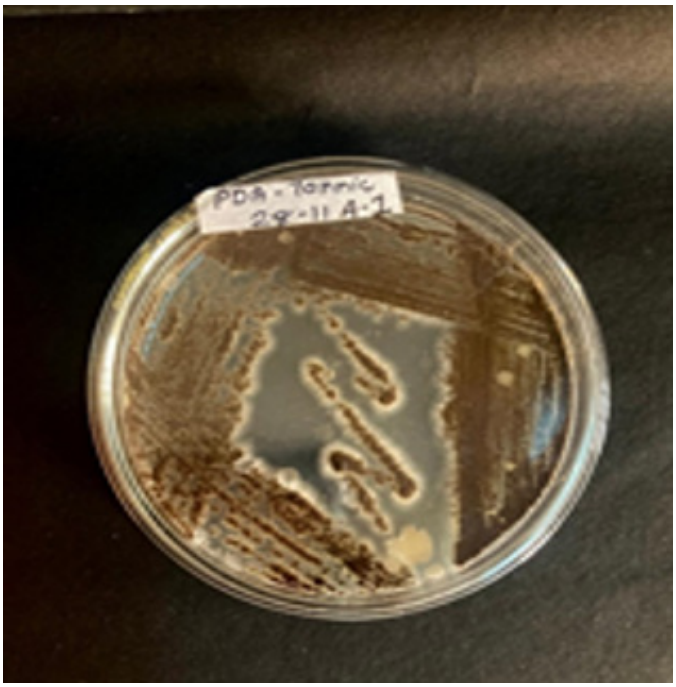

Fig. 2 Fungal Isolate Aspergilus spp

For fungi, the potent strain was initially grown in Olga medium, a submerged medium, showing high laccase output. Enzyme activity was monitored daily post-incubation, with significant activity (1.12 U/ ml) observed by day 6. However, due to a lack of specific inducers, the activity remained relatively low. Further tests revealed that fungi grew faster and produced more biomass on solid substrates compared to submerged conditions. The organism, grown on rice bran, demonstrated higher enzyme activity (12.1 U/ml) by the sixth day of incubation. Fungal laccase activity in solid medium extracts was notably higher than in liquid media extracts.

Decolorization of Synthetic Dyes: In the current investigation, Congo red dye was decolorized by laccase-producing bacterial and fungal isolates. The samples were taken out at regular intervals of two hours, and the degree of decolorization was determined spectrophotometrically using a UV-visible spectrophotometer. Congo red dye decolorization was successfully observed by at 560 nm, the absorbance was measured as per the graph.

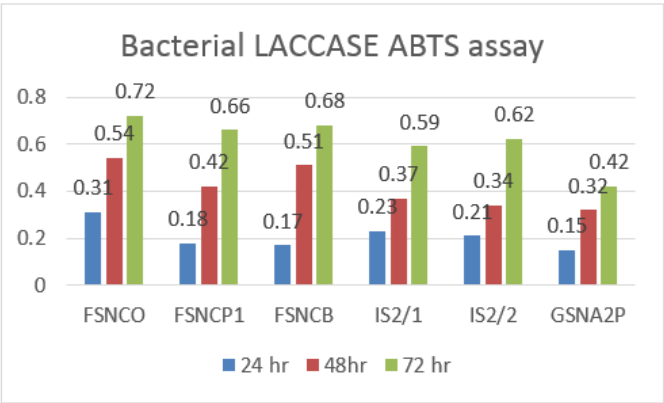

Fig.3 Graphical data of Bacterial Laccase GUAIACOL activity by Submerged Fermentation

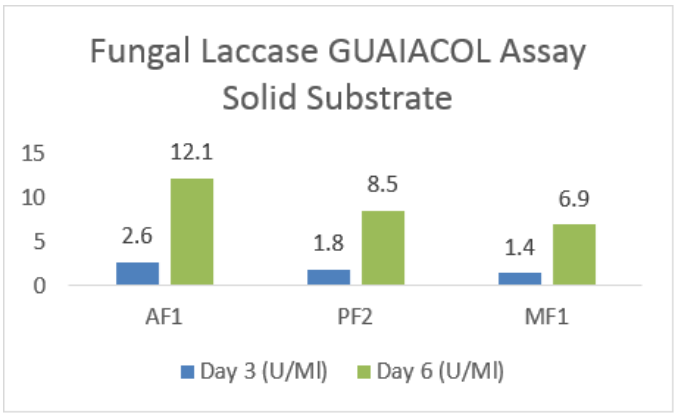

Fig.4 Graphical data of Fungal Laccase Enzyme GUAIACOL activity by using Solid Fermentation

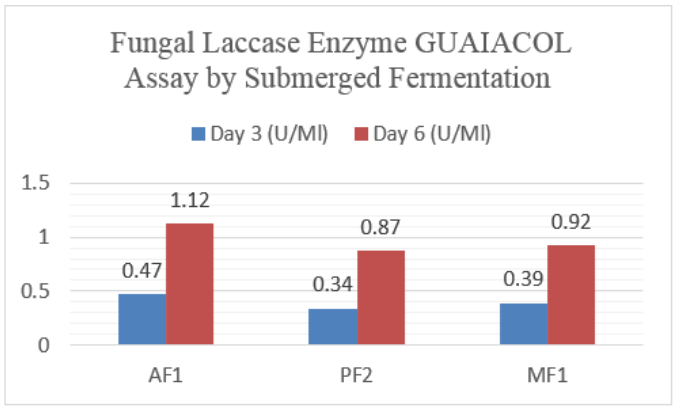

Fig.5 Graphical data of Fungal Laccase Enzyme GUAIACOL activity by using Submerged Fermentation.

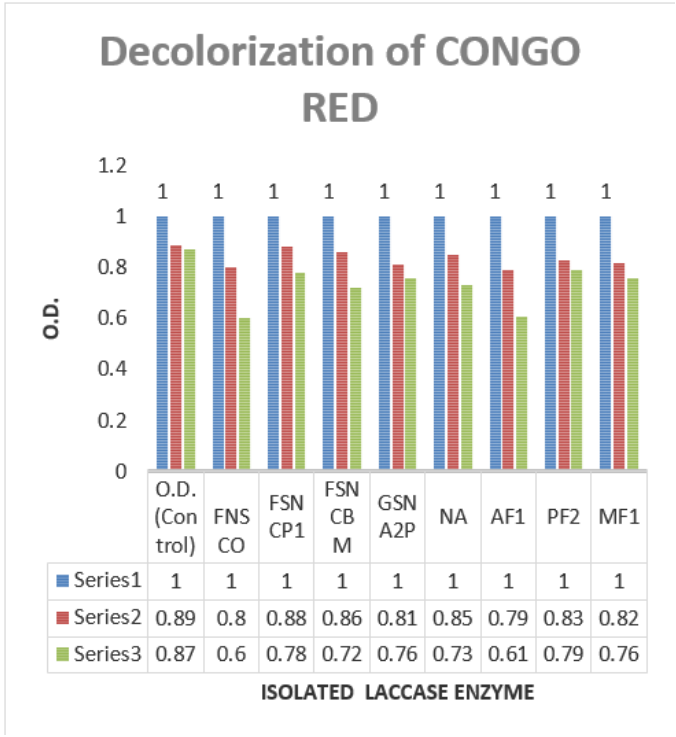

Fig. (6) Graphical data of Decolorization of Congo Red.

DISCUSSION

With important industrial impact, the current work provides

insights into enhancing laccase production employing bacterial and fungal isolates. In contrast with previous research, our bacterial isolate FSNCO had the maximum laccase activity (0.72 U/ml) over a 72-hour incubation period at 30°C. Chen et al. (2022) demonstrated strong production under a variety of circumstances, while Gupta et al. (2021) found comparable activity (0.180-0.250 U/ml) in strains from industrial effluents. Lower activity (0.150-0.200 U/ml) in isolates from agricultural waste were discovered by Zhao et al. (2023), indicating the great potential and adaptability of our FSNCO isolate.

Also, our Aspergillus strain showed significantly higher laccase production in solid state fermentation using rice bran (12.1 U/ml) compared to submerged conditions (1.12 U/ml in Olga's medium). This is consistent with Sharma et al. (2022) who reported an activity of 10.5-11.8 U/ml in Aspergillus strains on different substrates. These results highlight the efficiency of using solid-state fermentation for fungal laccase production, which may be due to better oxygen transfer and nutrient availability in the solid matrix.

The significant differences in laccase activity between solid state and immersion fermentations highlight the importance of optimizing fermentation conditions according to the specific microbial strain and substrate used. The high activity observed in the FSNCO isolate together with the improved solid-state fermentation production of the Aspergillus strain indicates promising applications in biotechnological processes including biorefining, biofuel production and various industrial applications where laccase enzymes play a crucial role.

Future studies should concentrate on improving fermentation conditions, investigating substitute substrates for more affordable production, and genetic engineering to increase laccase production. Furthermore, co-culturing fungal and bacterial strains may result in synergistic effects that boost enzyme activity even further. It will also be essential to look at industrial uses like bioremediation and biofuel production. The practical uses and economic viability of the production process can be improved by expanding it and incorporating it into industrial workflows.

CONCLUSIONS

The study highlights the potential of high laccase-producing bacteria Psuedomonas spp. from soap manufacturing waste for sustainable dye decolorization due to their adaptability to diverse environments. Further research is needed to optimize the production process and explore the use of agricultural as well as industrial wastes as substrates to enhance commercial viability.

Also, isolated Aspergillus strain, identified for its rapid growth and high laccase production, shows promise for large-scale production and industrial effluent treatment, particularly in solid-state conditions using rice bran. Optimization of production conditions is necessary for maximizing laccase yield and commercial application.

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